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(54) **Process for the production of a pullulan composition**

(57) A process for the production of a pullulan composition having a narrow molecular weight distribution, comprises partially hydrolysing a pullulan, fractionating the resulting pullulan partial hydrolysate, and collecting the fraction having Mw/Mn ratio not exceeding 1.5. The hydrolysis may be carried out using an acid catalyst, for example lactic, citric hydrochloric or sulphuric or by enzymatic treatment with, for example cyclodextrin glucanotransferase, α-amylase, pullulanase, isopullulanase or isoamylase, or by ultrasonic treatment. The partially hydrolyzed product may be separated by fractional precipitation using an organic solvent, for example methanol, ethanol or acetone or by fractional dissolution. The products are suitable on film formers the food industry and as a plasma extender.

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SPECIFICATION

Process for the production of a pullulan composition

Pullulan is a glucan which substantially consists of maltotriose units polymerised in a linear fashion via α-1,6-glucosidic linkages. Pullulan is generally obtained by cultivation of microorganisms of Aureobasidium pullulans in a medium containing saccharides, such as mono- and/or oligosaccharides, under submerged conditions.

Pullulan of a molecular weight of 80,000 - 300,000 has been produced on an industrial-scale and supplied for various uses. In particular certain properties of pullulan, such as its water-solubility, edibleness, adhesiveness and easy formability into film, make it attractive for use in the food and chemical industries.

With a view to enlarging the demand for this material, we have studied further the molecular weight distribution of commercial pullulan having various weight-average molecular weights (abbreviated as "$\bar{M}w$" hereinafter) by means of gel filtration to determine the weight-average molecular weight to number-average molecular weight ratio (abbreviated as "$\bar{M}w/\bar{M}n$" hereinafter). The study confirmed that all commercial pullulan tested had a relatively wide molecular weight distribution which was higher than about 2.0.

We have investigated processes for producing a pullulan composition having a narrow molecular weight distribution, in particular having a $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5.

Accordingly, the present invention provides a process for the production of a pullulan composition having a narrow molecular weight distribution, which process comprises partially hydrolysing pullulan, fractionating the resulting pullulan partial hydrolysate, and collecting the pullulan fraction having a $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5.

Our studies have led to the following conclusions: (1) Upon partial hydrolysis of pullulan, the higher the molecular weight of a fraction in the material, the more susceptible to hydrolysis is that fraction, and (2) the desired pullulan preparation having a narrow molecular weight distribution can be easily obtained in a higher yield by fractionation of a pullulan partial hydrolysate with a precipitant such as an organic solvent.

As regards the pullulan which is suitable for use in the invention, any pullulan preparation can be used as long as the desired pullulan preparation with $\bar{M}w/\bar{M}n$ not exceeding 1.5 can be obtained from its partial hydrolysate: usually, a pullulan having a higher $\bar{M}w$ than that of the desired pullulan preparation is suitable.

Suitable conditions for the partial hydrolysis are those whereunder the partial hydrolysis of the pullulan is effected, and the yield of the desired pullulan preparation is further enhanced: for example, the hydrolysis is attainable by treating an aqueous material pullulan solution with an organic or inorganic acid, such as lactic acid, citric acid, hydrochloric acid or sulfuric acid; or an enzyme such as cyclodextrin glucanotransferase (EC 2.4.1.19), α-amylase (EC 3.2.1.1.), pullulanase (EC 3.2.1.41), isopullulanse (EC 3.2.1.57) or isoamylase (EC 3.2.1.68); or by means of an ultrasonic device. The use of acid or enzyme hydrolysis is especially preferable in view of industrial-scale production.

The collection of the desired pullulan fraction from the partial hydrolysate can be performed usually by either a fractional precipitation using a water-soluble organic solvent, such as methanol, ethanol, isopropanol and acetone, or by using fractional dissolution, if necessary, in combination with other procedure(s), such as gel filtration and/or separation with a membrane filter.

The pullulan fraction thus obtained can be usually purified by decolourisation with activated carbon, and/or deionization with an ion exchanger. A colourless, transparent syrup can be easily obtained by membrane filtration and concentration; and a white, pyrogen-free pullulan powder, by membrane filtration, concentration, drying, and, if necessary, pulverisation or grinding.

Since the pullulan preparation thus obtained consists of highly-purified, water-soluble, linear polymer chains with $\bar{M}w/\bar{M}n$ not exceeding 1.5, it can be favourably used as an authentic water-soluble polymer: for example, in gel filtration or liquid chromatography, a pullulan preparation with $\bar{M}w/\bar{M}n$ of 1.1 to 1.3 is especially preferable. For such purposes, a standard water-soluble polymer kit providing various pullulan preparations of known $\bar{M}w/\bar{M}n$, for example, 20,000, 30,000, 40,000, 50,000 and 80,000, is conveniently usable.

The pullulan obtained according to the invention finds various uses as a plasma expander or a hyperkinemic: such compositions can be prepared by dissolving a pullulan having a $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5 ($\bar{M}w$ = 30,000 – 90,000) to give an aqueous pullulan solution with a concentration of about 4-10 w/v % on dry solid basis, adding an isotonic agent, such as mineral and/or saccharide, to the solution, and finally sterilizing the resulting isotonic pullulan solution.

The following Experiments further illustrate the present invention.

EXPERIMENT 1. Preparation of a pullulan having a narrow molecular weight distribution.

Three aliquots of a 10 w/v % aqueous pullulan solution with $\bar{M}w/\bar{M}n$ of 2.5 ($\bar{M}w$ = 150,000) were treated as follows:

(A) one aliquot was subjected to further processing without hydrolysis; (B) another aliquot was acidified to about pH 2 with sulfuric acid, and allowed to stand at this pH level and 80°C for two hours to effect partial hydrolysis, and after the hydrolysis the solution was rapidly neutralized; and (C) to the remaining aliquot was added a commercial α-amylase "NEOSPITASE" (Nagase & Company, Ltd., Osaka, Japan) in an amount of 550 dextrinogenic units per g pullulan, the mixture was incubated at pH 5.5 and 60°C for twenty hours to effect enzymatic hydrolysis, and then heated to inactivate the residual enzymatic activity. Then, to all aliquots (A), (B), and (C) methanol was added to give a respective concentration of 43 v/v %. The samples were kept at 30°C, and after the removal of the result-

ing lower-layers, the methanol was added to the remaining upper layers to give a respective concentration of 60 v/v %. After allowing the samples to stand, the newly-formed lower-layers were all collected, and ground to yield pullulan having a $\bar{M}w/\bar{M}n$ ratio of 1.4 ($\bar{M}w$ = 60,000).

The yields against the starting material pullulan are given in TABLE 1.

TABLE 1

Treatment	Non-hydrolysis	Hydrolysis	
		Acid	Enzyme
Yield (%)	16	48	55

From the results shown in TABLE 1, it will be seen that a larger amount of the desired pullulan can be obtained by collecting it from the partial hydrolysate than from the intact pullulan.

To obtain an explanation of these results, the distribution of molecular weights was determined by gel filtration on small portions of the hydrolysate which were collected at given intervals during the partial hydrolysis. The results confirmed that in partial hydrolysis using acid or enzyme, the higher the molecular weight of a particular fraction in the pullulan, the more susceptible to hydrolysis is that fraction. Thus, an appropriate selection of hydrolytic conditions results in a maximum yield of the desired pullulan, up to ca. 40-60%.

The results also establish that a partial hydrolysis with an enzyme gives a relatively higher yield than hydrolysis with an acid.

EXPERIMENT 2. Intravenous injection of pullulans with various $\bar{M}w/\bar{M}n$.

Four plasma expander solutions for intravenous injection test were prepared by dissolving aliquots of pullulan having respective $\bar{M}w/\bar{M}n$ ratios of 2.8, 2.0, 1.5 and 1.2 ($\bar{M}w$ = 60,000) into physiological saline solution to give a concentration of 6 w/v %, and sterilizing the resulting aliquot solutions.

The plasma expanders were each rapidly injected intravenously into rabbits, weighing about 3.0 kg, in dosages of 100 ml per kg within about 15 minutes, and then observations were made on the venous pressure and urinal excretion: venous (mmH_2O) before and after the injection were determined, and the ratio obtained by dividing the venous pressure after the injection by that before the injection, were used as a criteria for evaluating the effort of the circulatory system. In addition, the amount of pullulan excreted in the urine within two hours after the injection was determined, and the excretion ratios (%), expressing the ratio of the amount of the excreted pullulan to that of the injected pullulan, were used as a criterion for evaluating the circulating pullulan and its plasma expanding effect.

The experimental results are given in TABLE 2, wherein all values are mean values from experiments with two rabbits.

TABLE 2

$\bar{M}w/\bar{M}n$	2.8	2.0	1.5	1.2
Venous pressure (magnitude)	2.1	1.7	1.3	1.2
Excretion ratio (%)	45	32	17	13
Weight	(−)*	(±)*	(+)**	(+)**

Note: (+) = superior; (±) = normal; and (−) = inferior
*) = control; and **) = present invention.

From the results shown in TABLE 2 it will be seen that the use of pullulan having a $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5 results in a slight venous pressure increase, and not in a rapid urinal excretion of the injected pullulan; such pullulan is therefore very suitable for plasma expanding.

Then, a pullulan with $\bar{M}w/\bar{M}n$ of 2.8 ($\bar{M}w$ = 60,000) was fractionated by gel into two specimens; one specimen had an average molecular weight not exceeding 15,000 and the other, higher than 150,000; these were separately injected into rabbits in a manner similar to that described above. The injection of the first specimen resulted in an about 1.2-fold venous pressure increase, but in an up to 80% urinal excretion; in contrast, the injection of the second specimen resulted in an about 7% urinal excretion, but in an up to about 3-fold venous pressure increase.

The results led to the following conclusion; in a pullulan with an average molecular weight not exceeding 15,000 the half time required from injection to urinal excretion is very short, resulting only in a higher effort of kidney, therefore no plasma expanding effect can be expected therewith. On the other hand, the use of a pullulan with an average molecular weight higher than 150,000 has fear for a relative venous pressure increase upon a rapid intravenous injection, therefore the effort of the circulatory system would increase extremely.

Accordingly, a pullulan suitable for use as a plasma expander should have a narrow molecular weight distribution with its $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5, obtained by decreasing both a lower molecular weight specimen (<15,000), and the higher molecular weight specimen (>150,000) as much as possible, in addition to bringing in a molecular weight of 30,000 - 90,000, as suggested in the Japanese Patent Application No. 63,976/79.

The present invention is further illustrated by the following Examples.

EXAMPLE 1

A 10 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.3 ($\bar{M}w$ = 300,000) in water, was acidified to about pH 2 with hydrochloric acid, incubated at 80°C for

two hours to effect partial hydrolysis, and then neutralised with sodium hydroxide.

Thereafter, the pullulan partial hydrolysate solution was cooled to 30°C, methanol was added to give concentration of 40 v/v % while maintaining the temperature. After removal of the resulting lower-layer, methanol was added to the remaining upper-layer to give a concentration of 55 v/v %, and the mixture was then allowed to stand, and the newly-formed lower-layer was then collected.

Then, methanol was removed from the lower-layer by distillation, and the residual aqueous pullulan solution was purified by decolourisation with activated carbon, deionised with H- and OH-form ion exchangers, and subjected to membrane filtration. The resultant material was concentrated, dried, and ground to give about 90 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.4 ($\bar{M}w$ = 50,000).

EXAMPLE 2

A 20 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.6 ($\bar{M}w$ = 80,000) in water, was acidified to about pH 2 with sulfuric acid, and then subjected to partial hydrolysis at 80°C for two hours, followed by neutralisation of the resulting pullulan partial hydrolysate solution with sodium hydroxide.

Thereafter, ethanol was added to the solution to give a concentration of 50 v/v % while maintaining at 40°C, and after removal of the resulting lower-layer, further ethanol was added to the remaining upper-layer was added to give a concentration of 70 v/v %. The newly-formed lower-layer was then collected.

The lower-layer was purified in a manner similar to that described in Example 1, to give about 70 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.3 ($\bar{M}w$ = 30,000).

EXAMPLE 3

A 5 w/v % aqueous solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.3 ($\bar{M}w$ = 200,000) in water, was partially hydrolysed in a manner similar to that described in Example 1, followed by neutralisation.

Acetone was added to the pullulan partial hydrolysate solution to give a concentration of 20 v/v %, and after removal of the resulting lower-layer, further acetone was added to the remaining upper-layer to give a concentration of 45 v/v %.

The newly-formed lower-layer was collected, and purified in a manner similar to that described in Example 1 to give about 80 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.5 ($\bar{M}w$ = 85,000).

EXAMPLE 4

To a 10 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.3 ($\bar{M}w$ = 300,000) in water, was added a cyclodextrin glucanotransferase (EC 2.4.1.19), as disclosed in the Japan Patent Publication No. 27,791/78, in an amount of 150 dextrinogenic units per g pullulan. The mixture was subjected to an enzymatic hydrolysis at 65°C and pH 6.0 for twenty hours. After the hydrolysis, the enzymatic reaction was suspended by heating at 90°C for 15 minutes.

The resulting pullulan partial hydrolysate solution was fractionated with methanol, and the desired fraction was purified, concentrated and ground in a manner similar to that described in Example 1 to give about 105 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.4 ($\bar{M}w$ = 50,000).

EXAMPLE 5

To a 20 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.6 ($\bar{M}w$ = 80,000) in water, was added a commercial pullulanase (EC 3.2.1.41), a product of Hayashibara Biochemical Laboratories Inc., Okayama, Japan, in an amount of four units per g pullulan. The mixture was subjected to an enzymatic hydrolysis at pH 6.0 and 50°C for thirty hours. After suspending the enzymatic reaction by heating, the pullulan partial hydrolysate solution was fractionated with ethanol, and the desired fraction was purified, concentrated, and ground in a manner similar to that described in Example 2 to give about 80 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.1 ($\bar{M}w$ = 20,000).

EXAMPLE 6

To a 5 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.1 ($\bar{M}w$ = 200,000) in water, was added a commercial α-amylase "NEOSPITASE" (Nagase & Company, Ltd., Osaka, Japan) in an amount of 500 dextrinogenic units per g pullulan. The mixture was incubated at pH 6.4 and 55°C for 24 hours to effect an enzymatic hydrolysis. After stopping the hydrolysis by heating, the resulting pullulan partial hydrolysate solution was fractionated with acetone, and the desired fraction was purified, concentrated, and ground in a manner similar to that described in Example 3 to give about 110 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.5 ($\bar{M}w$ = 85,000).

EXAMPLE 7

To a 10 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.3 ($\bar{M}w$ = 200,000) in water, was added a commercial isoamylase (EC 3.2.1.68), a product of Hayashibara Biochemical Laboratories Inc., Okayama, Japan, in an amount of 200 dextrinogenic units per g pullulan. The mixture was incubated at pH 4.3 and 50°C for 20 hours to effect an enzymatic partial hydrolysis.

After stopping the hydrolysis by heating, the resulting pullulan partial hydrolysate solution was fractionated with methanol, and the desired fraction was purified, concentrated, and ground in a manner similar to that described in Example 1 to give about 90 g of white pullulan powder having $\bar{M}w/\bar{M}n$ ratio of 1.2 ($\bar{M}w$ = 40,000).

EXAMPLE 8

A 10 w/v % aqueous pullulan solution, prepared by dissolving 200 g pullulan having a $\bar{M}w/\bar{M}n$ ratio of 2.1 ($\bar{M}w$ = 580,000) in 0.05 M NaCl solution, was treated with an ultrasonic device "BRANSON ULTRASONIC CLEANER Model 12", Registered Trade Mark of Yamato Scientific Co. Ltd., Tokyo, Japan, for 30 minutes at 45 KHz, output power, 50 W, to effect the partial hydrolysis of the pullulan.

The resulting pullulan partial hydrolysate solution was fractionated with methanol, and the desired fraction was purified, concentrated, and ground in a manner similar to that described in Example 1 to give about 70 g of white pullulan powder having a $\bar{M}w/\bar{M}n$ ratio of 1.4 ($\bar{M}w$ = 40,000).

CLAIMS

1. A process for the production of a pullulan composition having a narrow molecular weight distribution, which process comprises partially hydrolysing pullulan, fractionating the resulting pullulan partial hydrolysate, and collecting the pullulan fraction having a $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5.

2. A process according to Claim 1, wherein the partial hydrolysis of the pullulan is carried out with an acid selected from lactic acid, citric acid, hydrochloric acid and sulfuric acid.

3. A process according to Claim 1 or 2, wherein the partial hydrolysis of the pullulan is carried out by an enzymatic action of enzyme selected from cyclodextrin glucanotransferase, α-amylase, pullulanase, isopullulanase and isoamylase.

4. A process according to Claim 1, 2 or 3, wherein the partial hydrolysis of the pullulan is carried out ultrasonically.

5. A process according to any one of the preceding claims, wherein the fractionation is carried out by means of fractional precipitation using an organic solvent selected from methanol, ethanol, isopropanol and acetone.

6. A process according to any one of Claims 1 to 4, wherein the fractionation is carried out by means of fractional dissolution.

7. A process according to Claim 1 substantially as described in any one of the foregoing Examples and Experiments.

8. A pullulan composition having a $\bar{M}w/\bar{M}n$ ratio not exceeding 1.5 when prepared by a process as claimed in any one of the preceding claims.

9. A pullulan composition according to Claim 8 having a $\bar{M}w$ from 30,000 to 90,000.

10. A plasma expander incorporating a pullulan composition as claimed in Claim 8 or 9.

11. A hyperkinemic incorporating a pullulan composition as claimed in Claim 8 or 9.

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TI Pullulan blood plasma extender mfr. - by partial decomposition of raw pullulan and fractionating

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L2 ANSWER 2 OF 2 CAPLUS COPYRIGHT 2007 ACS on STN

Full Text | Citing References

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TI Pullulan preparation with limited molecular weight distribution for use as a plasma extender and an antihyperkinesis agent

IN Yoshida, Mikihiko

PA Hayashi Biochemical Laboratories, Inc., Japan

SO Ger. Offen., 16 pp.
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